Diagnosis of Adrenal Cortical Dysfunction by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to measure 6 metabolic compounds of the adrenocorticosteroid pathway simultaneously on residual specimens from patients who had previously been previously diagnosed, on the basis of immunoassays, as having congenital adrenal hyperplasia (CAH), 11β-hydroxylase deficiency, 21-hydroxylase deficiency, or Addison disease (adrenal insufficiency). Two subjects with normal adrenal function had serum cortisol values of 13.6 and 8.9 µg/dL and serum cortisone values of 2.1 and 0.6 µg/dL, but the rest of the compounds were undetectable. Two patients with 11β-hydroxylase deficiency had serum 11β-deoxycortisol values of 14.9 and 10.0 µg/dL and serum 11-deoxycorticosterone values of 3.9 and 1.0 µg/dL, but their serum levels of cortisol and cortisone were diminished. A patient with 21-hydroxylase deficiency had a highly increased serum 17-hydroxyprogesterone concentration of 28.5 µg/dL (or 28,500 ng/dL, the traditional unit to report this assay) and a serum 21-deoxycortisol concentration of 6.9 ug/dL (this is a pathologic marker of 21-hydroxylase deficiency that is nondetectable in sera of healthy subjects). This patient also had diminished concentrations of serum cortisol and cortisone (0.9 and 0.3 µg/dL, respectively). At 30 and 60 min after corticotropin (ACTH) stimulation, serum cortisol was the only compound that showed a dramatic increase in the normal subjects; the patient with 21-hydroxylase deficiency showed an increase of serum 17-hydroxyprogesterone level, but no increase of serum cortisol level; the patient with Addison disease showed no increase in the levels of serum cortisol or other compounds. Metyrapone, which blocks 11β-hydroxylase activity, increased the serum 11-deoxycorticosteroid levels and decreased the serum cortisol level. This pilot study demonstrates that it is feasible to use LC-MS/MS for the laboratory diagnosis of adrenal cortical dysfunction. The authors envision that LC-MS/MS may soon become an ideal analytical technique for the diagnosis of such endocrine diseases. (received 1 November 2000, accepted 6 February 2001)

Keywords: Liquid chromatography-tandem mass spectrometry, serum steroid analysis, adrenal cortical dysfunction

Introduction

New analytical technologies will change clinical laboratory operations in the new millennium. In an article on future trends in clinical chemistry, Parker [1] recently commented that “flow cytometry and mass spectrometry are currently peripheral; they will become mainstream technologies tomorrow.” A pertinent question is, When is the tomorrow? This pilot study suggests that the tomorrow is very soon and may be within the next five years. Currently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is available in only a few medical centers. To make LC-MS/MS a mainstream technology that will benefit the entire medical community, instrument manufacturers should take the initiative to introduce relevant clinical tests for this instrumentation and to demonstrate its practical clinical applications.

A good candidate for such applications is the use of LC-MS/MS to obtain multiple test results for complicated adrenal disorders. Performing multiple analyses of hormones in a single determination has long been a goal of our laboratory. In the past, many clinically useful endocrine tests were developed that

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0091-7370/01/0200/0197 $1.50; © 2001 by the Association of Clinical Scientists, Inc.
provided multiple analyses (eg, 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, cortisol and cortisone, and the catecholamines) by high-performance liquid chromatography (HPLC). In these methods, only 2 or 3 analytes could be detected using spectrometric or amperometric detectors [2–4]. LC-MS/MS can be considered as an HPLC apparatus that is equipped with a mass spectrometric detector that measures the mass and charge ratios (m/z) of analytes and their fragments. Numerous analytes can now be measured simultaneously by LC-MS/MS with positive identification.

Immunoassays lack positive identification, even with the use of specific antibodies, and occasionally they can give equivocal results owing to cross-reactivities, or the presence of human anti-animal antibodies in a patient’s serum [5–7]. In particular, modern, highly sensitive, immunometric assays, which require no preanalytical extraction to concentrate and purify the analyte, tend to magnify the problems of nonspecificity and interferences.

Because LC-MS/MS can provide multiple determinations in a single step, it simplifies a laboratory’s operation and helps to avoid an excessive amount of blood being drawn from a patient [8]. All of the critical steroid metabolites in serum can be analyzed simultaneously in a single determination, and the frequency and volume of blood collection are substantially reduced.

The LC-MS/MS technology is also economical, as demonstrated by cost accounting studies of other assays. In a study of homocysteine assays, the relative costs per test, including supplies and equipment, using LC-MS/MS, HPLC, or a commercial immunoassay kit, were estimated to be 1.00, 1.15, and 5.08, respectively [9]. Cost-effectiveness of LC-MS/MS was also demonstrated in assays of methylmalonic acid [10].

Materials and Methods

Specimens for analysis. This study used residual specimens of serum or plasma (0.5 mL) from patients who had previously been diagnosed, on the basis of immunoassays, as having adrenal disorders (eg, 11β-hydroxylase deficiency, 21-hydroxylase deficiency, Addison disease). Morning fasting serum specimens from 36 healthy subjects, including 11 men (28 to 68 yr) and 25 women (25 to 61 yr), were also analyzed. This project was approved by the Mayo Clinic’s Institutional Review Board.

Instrumentation and reagents. The tandem mass spectrometer (API 2000, PE Biosystems, Foster City, CA) included an electrospray ionization device, a triple-quadruple mass spectrometer, and an electron multiplier detector. Cortisol, cortisone, 17-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, 11-deoxycorticosterone were from Sigma Chemical Co. (St. Louis, MO). Deuterium-labeled cortisol (cortisol-9,-11,-12,-12,-d4) was from Cambridge Isotope Labs (Andover, MA). HPLC grade methanol and methylene chloride were from Fisher Scientific (Pittsburgh, PA). Steroid stock standards were prepared in 100% methanol and stored at -20°C. The reverse-phase chromatographic column was “Supelcosil LC-18” (33 x 4.6 mm) from Supelco (Bellefonte, PA).

Sample preparation. Serum or plasma (0.5 mL) was placed in a 10 x 75-mm glass tube and mixed with 7 µl of 10 µg/ml cortisol-d4. For extraction, methylene chloride (4.5 mL) was dispensed into each tube; the tube was capped, mixed gently on a rotating mixer for 30 min, and centrifuged at 2500 rpm for 5 min. The upper aqueous layer was removed by aspiration; the lower organic phase was evaporated to dryness under nitrogen gas at 45°C. The dried extract was reconstituted with 125 µL of a 70:30 (v/v) methanol-water mixture, which was transferred to a screw-capped injection vial. A 12-point calibration curve containing each steroid to be tested, with concentrations from 0.5 to 25 µg/dL, was prepared prior to analysis. A 17-µL injection volume was used for standards and samples.

LC-MS/MS analysis. Reverse-phase LC, which separated each steroid in the sample, involved a 10-min analysis with a linear gradient (53% to 75%) of methanol-water at a flow rate of 1 mL/min. The chromatographic column was connected directly to the ion source of the “TurbolonSpray” (PE Biosystems) for ionization probe; the column effluent flow was split to deliver approximately 100 µL/min to the mass spectrometer. The instrument was set to use 3 separate scan periods over a 10-min running time. The positive ion mode was used throughout the analysis. The following software was used: “Mass-Chrom” (version
1.11) and “Multi-View” (version 1.4) for chromatographic and spectral interpretations, and "Turbolon-Quan" (version 1.0, PE Biosystems) for data processing on a Macintosh computer [11]. The sensitivity for cortisol detection using d4-cortisol calibration was 0.1 µg/dL. The sensitivities of the other metabolites were 0.5 µg/dL. The assay CV was <5%, similar to HPLC.

### Results

**Healthy subjects.** Of 2 healthy subjects, one had serum cortisol and cortisone values of 13.6 µg/dL and 2.1 µg/dL, respectively. The other had a serum cortisol value of 8.9 µg/dL, a serum cortisone value of 0.6 µg/dL, and a trace of 11-deoxycortisol (<0.5 µg/dL). In serum specimens from 36 healthy subjects, the cortisol concentration (mean ± SD) was 14.3 ± 8.1 µg/dL (range 2 to 30 µg/dL). The cortisone concentration was 2.2 ± 0.6 µg/dL (range 0.2 to 3.2 µg/dL). The remaining steroid compounds were undetectable in serum specimens from healthy subjects.

**11 β-Hydroxylase deficiency.** Two patients with 11 β-hydroxylase deficiency had only trace amounts of serum cortisol (0.5 µg/dL) and undetectable amounts of cortisone, but they had high levels of 11 β-deoxycortisol (14.9 and 10.0 µg/dL, respectively). These patients had serum 11-deoxycorticosterone values of 3.9 and 1.0 µg/dL and measurable amounts of serum 17-hydroxyprogesterone (0.9 and 1.2 µg/dL) (Table 1).

**21-Hydroxylase deficiency.** A patient with 21-hydroxylase deficiency had a high level of serum 17-hydroxyprogesterone (28.5 µg/dL, ie, 28,500 ng/dL) and trace amounts of cortisol (0.9 µg/dL) and cortisone (0.3 µg/dL). A unique compound, 21-deoxycortisol, is found only in serum from patients with 21-hydroxylase deficiency. 21-Deoxycortisol is a marker of 21-hydroxylase deficiency and is not a normal precursor in the pathway of adrenocortical hormone biosynthesis. The concentration of serum 21-deoxycortisol in this patient was 6.9 µg/dL. Trace amounts of 11-deoxycortisol and DOC also were present in this patient’s serum (0.9 µg/dL and 0.3 µg/dL, respectively) (Table 1).

**ACTH stimulation test.** Fig. 1 shows illustrative data for two patients who gave normal responses during the (1-24) ACTH stimulation test. Both patients had increases of serum cortisol levels, which peaked at 30 or 60 min after the injection of cosyntropin (0.5 mg/patient). The serum concentrations of precursors in the glucocorticoid and mineral corticoid pathways showed little change (Fig. 1). In contrast, a patient with Addison disease, whose baseline ACTH level was 1,900 pg/mL (reference range, <23 pg/mL), showed

<table>
<thead>
<tr>
<th>Test subjects</th>
<th>Cortisol</th>
<th>Cortisone</th>
<th>11 β-Deoxy cortisol</th>
<th>Deoxycorticos- terone (DOC)</th>
<th>17-Hydroxy- progesterone</th>
<th>21-Deoxcortisol</th>
</tr>
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<tbody>
<tr>
<td>Healthy subjects&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subject #1</td>
<td>13.6 (19)</td>
<td>2.1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Subject #2</td>
<td>8.9</td>
<td>0.6</td>
<td>trace</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>11 β-Hydroxylase deficiency</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Patient #1</td>
<td>0.5 (4.7)</td>
<td>nd</td>
<td>14.9 (15)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.9</td>
<td>0.9</td>
<td>nd</td>
</tr>
<tr>
<td>Patient #2</td>
<td>0.5 (3.9)</td>
<td>nd</td>
<td>10.0 (13)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>1.2</td>
<td>nd</td>
</tr>
<tr>
<td>21-Hydroxylase deficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient #1</td>
<td>0.9 (7.0)</td>
<td>0.3</td>
<td>0.9</td>
<td>0.3</td>
<td>28.5 (15.9)</td>
<td>6.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> The numbers in parentheses are results that were previously obtained by immunoassays to establish the diagnoses. The reference values for the immunoassays are: cortisol (am specimens), 7 to 25 µg/dL; deoxycorticosteroids (a combined measurement of 11 β-deoxycortisol and DOC)<sup>c</sup>, 0 to 5 µg/dL; 17-hydroxyprogesterone, <0.2 µg/dL.

<sup>b</sup> Assays of sera from 36 healthy subjects (fasting morning specimens) gave the following results (mean ± SD): cortisol 14.3 ± 8.1 µg/dL; cortisone 2.2 ± 0.6 µg/dL; the other four steroid compounds were “nd” (not detected).
no change in the serum levels of cortisol or its precursors at 30 or 60 min after an ACTH injection (Fig. 2). In the patient with 21-hydroxylase deficiency, serum 17-hydroxyprogesterone concentration was 5.1 µg/dL at baseline and increased to 21.3 µg/dL at 30 min after the ACTH injection and 20.3 µg/dL at 60 min. The serum cortisol value remained unchanged (4.9 µg/dL) during this period. This patient's ACTH stimulation test was performed at the beginning of our study, before we could measure serum 21-deoxycortisol.

Metyrapone test. In two patients, overnight metyrapone tests were performed by administering 3 g of metyrapone at 11 pm in order to block 11 beta-hydroxylase activity. Serum obtained on the next morning had increased 11-deoxycortisol and decreased cortisol levels. In one patient, serum cortisol decreased from 5.1 to 1.3 µg/dL, 11-deoxycortisol increased from 0.6 to 12.5 µg/dL, and DOC increased from undetectable...
to 3.9 µg/dL. In the other patient, serum cortisol decreased from 4.4 to 2.1 µg/dL, 11-deoxycortisol increased from 0.6 to 10.4 µg/dL, and DOC increased from undetectable to 1.8 µg/dL (Fig. 3).

**Discussion**

The authors predict that LC-MS/MS will soon become a mainstream technology for the diagnosis of adrenal cortical dysfunction, since multiple compounds of the steroid-biosynthetic pathway can be measured simultaneously in a single serum analysis. Availability of this technology will make differential diagnosis of enzyme deficiency in congenital adrenal hyperplasia (CAH) simpler than the customary procedure of ordering multiple immunoassays of individual metabolites according to algorithms or diagnostic flow charts. Many endocrinologists, geneticists, and pediatricians are enthusiastic about this new method, because by a single test they can detect the phenotype of congenital adrenal enzyme deficiency. They can readily make a complete diagnosis without referring the patient for time-consuming confirmatory studies.

Economics will be a factor that either delays or accelerates the introduction of LC-MS/MS for routine use in clinical laboratories. Although LC-MS/MS is generally viewed as an expensive analytical procedure, cost-comparison studies of assays for homocysteine and methylmalonic acid indicate that LC-MS/MS is actually less costly than HPLC or immunoassays, provided the test volume is large enough [9,10].

It is difficult to predict the costs of using LC-MS/MS to measure six steroid metabolites in a single determination, as in this study The economical use of mass spectrometry will require deuterium-labeled compounds as internal standards for each specific analyte, and such standards were unavailable to us. The use of non-labeled standards to prepare a 12-point standard curve is too inefficient and time-consuming. We envision that, in response to growing clinical demand, the biomedical industry will supply the requisite deuterium standards, as well as specific analytical and interpretative computer software. The price of instruments for tandem mass spectrometry should decrease with its increasing use.

Tandem mass spectrometry gives positive identification to each assayed metabolite and thus is superior to HPLC and immunoassays, which do not provide positive identification [8]. Immunoassays may cross-react with other compounds of the steroid pathway or with drugs of similar structure. A patient’s serum may contain human anti-animal immunoglobulins (HAMA), which also cause interference. The HAMA interference gives either falsely high or low results and may lead to an incorrect diagnosis [3,7,8].

This pilot study demonstrated the usefulness of tandem mass spectrometry for the differential diagnosis of 11 β-hydroxylase and 21-hydroxylase deficiencies and for the diagnosis of Addison disease. In regard to 21-hydroxylase (Cyp 21 A2) deficiency, patients with this condition have thousand-fold increase of serum nonconjugated 17-hydroxyprogesterone and their serum contains 21-deoxycortisol, which is a marker of the 21-hydroxylase deficiency. In addition, the concentrations of serum cortisol and cortisone are reduced. These results are obtained simultaneously in a single determination by LC-MS/MS. Other assays cannot accomplish this.

The corticotropin stimulation test with synthetic (1-24) ACTH revealed that the endocrine response was very rapid. When specimens were collected at 30 and 60 min after ACTH stimulation, cortisol was the only serum metabolite that increased in the normal subjects. Upstream intermediate compounds such as 11-deoxycortisol or 17-hydroxyprogesterone did not accumulate in serum at these intervals after ACTH injection; the precursors were converted to cortisol rapidly, within 30 min.

After pharmaceutical blocking of 11 β-hydroxylase by metyrapone, patients had decreases of serum cortisol and cortisone and increases of serum 11-deoxycortisol and DOC. This situation is similar to CAH (11-hydroxylase deficiency). LC-MS/MS enhances the ability to diagnose such enzyme deficiency. ACTH stimulates cholesterol side-chain cleavage by P450ccc enzyme (which, in the new genetic terminology, is CYP 11 A1), starting the pathway of steroid biosynthesis and increasing serum cortisol. Metyrapone blocks 11-hydroxylase (P450c, or CYP 11 B1) activity and inhibits the conversion of 11-deoxycortisol to cortisol. The data obtained after ACTH stimulation and metyrapone blockade of enzyme pathways indicate that LC-MS/MS analysis can facilitate pathway profiling to find new drugs that target specific enzymes.
References